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Inorganic Aspects of the Blood Chemistry of Ascidians. Ionic Composition, and Ti, V, and Fe in the Blood Plasma of *Pyura chilensis* and *Ascidia dispar*

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Abstract. Iron, titanium, and vanadium analysis were performed on the tunicates *Pyura chilensis* Molina, 1782, and *Ascidia dispar*, and the inorganic chemistry of blood was investigated. The major ionic characterization of the blood plasma and cytosolic solutions were determined. Gel chromatography was used to secure information on the possible existence of metal organic complexes.

Pyura chilensis accumulates Fe and Ti, and *Ascidia dispar* accumulates Fe, Ti, and V in blood cells in this quantitative order. Significant levels of metals are associated with cell residues (membrane cells), although this may be, to some extent, dependent on the cell lysis technique.

The elution behavior of plasma in Sephadex G-75 and LH-20 gels and the respective absorption spectra of the fractions showed evidence of organic metal complexes in the plasma of both tunicate species.

Introduction

For years tunicates have piqued the curiosity of biologists because of their unusual physiological peculiarities and because they may have given rise to the vertebrates (Berril, 1955). Among the physiological peculiarities that distinguish these organisms from others are the following: (i) They need a low tension of oxygen (Goodbody, 1974). To date, no reversible binding of oxygen has been detected nor the unequivocal existence of a proteic O_2 transport compound that transports O_2 through the blood (Macara *et al.*, 1979a; Agudelo *et al.*, 1982). (ii) They are entirely ammonotelic in their protein metabolism, but are uricotelic with respect to nucleic acid metabolism. Therefore, they differ from most invertebrates that are wholly ammonotelic, accumulating uric acid and purines in nephrocyte vacuoles (Goodbody, 1974; Wright, 1981). The functional importance of this storage remains obscure. (iii) They are capable of humoral and cellular immunological responses (Wright, 1981) and are rich in bio-active substances (Roman, 1986). (iv) They accumulate metal ions.

With respect to metal ions, tunicates are known for the uptake of selected metals from seawater and for accumulating them in their blood (Carlisle, 1968; Swinehart *et al.*, 1974; Senozan, 1974; Biggs and Swinehart, 1976). Members of the order Enterogona can accumulate vanadium (Kustin *et al.*, 1975; Kustin and McLeod, 1977; Macara *et al.*, 1979b; Biggs and Swinehart, 1979; Botte *et al.*, 1979; Dingley *et al.*, 1981; Hori and Michibata, 1981; Rowley, 1982; Dingley *et al.*, 1982). However, the type of coordination compound(s) in which the metal is involved in the blood is unknown (Carlson, 1975; Tullius *et al.*, 1980; Dingley *et al.*, 1982; Hawkins *et al.*, 1983a; Bruening *et al.*, 1985; Frank *et al.*, 1986). Members of the order Pleurogona, sub-order Stolidobranchiata, accumulate iron (Endean, 1955a, b, c; Agudelo *et al.*, 1982; Agudelo *et al.*, 1983a, b; Agudelo *et al.*, 1985), but nothing is known about its function in blood cells (Hawkins *et al.*, 1983b). In plasma, iron is associated with transferrin-like metalloproteins (Martin *et al.*, 1984; Finch and Huebers, 1986).

Hawkins *et al.* (1983c) proposed that ascidian taxonomy reflects a separation into vanadium- and iron-containing species. Tunicates accumulate other metals besides vanadium and iron (Monniot, 1978; Macara *et al.*, 1979c; Agudelo *et al.*, 1981; Rowley, 1982), which may

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not be essential elements subjected to selective accumulation mechanisms. Sessile filter feeding animals are very sensitive to their immediate environment, and significant amounts of contaminating metallic elements could be taken up by ascidians (Papadopoulou and Kanias, 1977).

In processes in which metals are accumulated in blood cells, it is logical that metals make a transient or permanent appearance in blood plasma. Once metals gain access to the body interior, they must be appropriately distributed, but because of its hydrolysis property some of these metals cannot be held in solution, in the interior media, without some mechanism to prevent its precipitation.

No metalloproteins such as hemocyanin have been reported in ascidian blood plasma. However, Hawkins *et al.* (1980a) and Webb and Chrystal (1981) studied the metal binding properties—including spectral characterization and metal contents—of some tunicates (Hawkins *et al.*, 1980b). They found preliminary evidence of metal complexing. This was confirmed by Martin *et al.* (1984) in the plasma of *Pyura stolonifera,* by demonstrating an iron-binding protein of about 40,000 daltons molecular weight with one iron-binding site considered as one *Pyura* transferrin (Finch and Huebers, 1986).

In this work, the Ti, V, and Fe contents were determined in several tissues. Also, the major characterization and chromatographic elutive behavior on Sephadex G-75 and LH-20 gels of *Pyura chilensis* Molina, 1782, and *Ascidia dispar* blood plasma were examined. These are two phylogenetically diverse ascidians.

Materials and Methods

Chemicals were from Merck. 3,3'-dimethylnaphthidine was from Eastman organic chemicals and ophenanthroline hydrochloride was from Riedel-De Haen. Sephadex G-75, LH-20 gels and blue dextran 2000 were from Pharmacia Fine Chemicals. Deionized water was prepared from distilled water passed through a disposable demineralizer cartridge (Corning 3508-B).

Specimens of *P. chilensis* and *A. dispar* were collected at Bahía Mejillones del Sur (Antofagasta-Chile) from marine pools, in which they were found as encrusting fouling organisms. *P. chilensis* afixes itself to ropes while *A. dispar* attaches itself to painted floating metallic barrels where they coexist with hydrozoans and bryozoans. Before drawing blood, specimens were maintained for some time in seawater at room temperature, and then were gently squeezed to remove most of the seawater.

Blood samples of both species were obtained by cutting the base of the body. Blood cells were removed from the plasma by centrifuging (2500 rpm; 10 min). Plasma was kept at 4–5°C while carried to the laboratory and was used as soon as possible.

Cellular residues presumably consisted of cell membranes. No distinction was made between cell surface and intracellular membranes. Cell samples were rinsed with seawater and then subjected to two different cell lysis processes. In the first procedure, cells were subjected to three freeze-thaw cycles in deionized water media (1.4 parts of triturated ice + 2 parts of $CaCl_2 \times 6H_2O$ freeze/room temperature), gently squeezed with a cell teflon homogenizer, and then centrifugated at 8000 rpm. In the second procedure cells were subjected twofold to an excess of methanolic solution of 0.75% HCl (Hawkins, pers. comm.) and centrifuged at 8000 rpm. In both cases the cytosolic solution and methanolic extract were made up to the original volume from which the cells were obtained. Whole blood samples of *P. chilensis* were subjected to the first cell lysis procedure, but without deionized water. A Sorvall refrigerated centrifuge was used.

Metal analysis

Prior to the Ti, V, and Fe determinations in specimens and tissues, a qualitative analysis was performed on digested blood cells. Cells rinsed with microfiltered seawater were digested with binary $HNO_3/HClO_4$ acid system (Jones *et al.*, 1982), performing assays for Cu, Mn, Fe, Ni, Co, Ti, V, and Nb (Feigl and Anger, 1972). Mn and Fe were also subjected to semi-quantitative assays with Merkoquant sticks.

Pyura chilensis and *Ascidia dispar* were analyzed individually. Tissues including blood were obtained from 10–20 specimens of *P. chilensis* and 30–50 specimens of *A. dispar.* Bodies were separated from tunics and rinsed with filtered seawater. Tunics were gently scrubbed with a plastic brush to remove dirt and rinsed in a similar manner. Siphons and tunics were cut off with a hard acrylic knife. Specimens and tissues, including some samples of plasma, cells, and cellular residues, were then dried at 110°C to constant weight, digested with a binary acid procedure (Jones *et al.*, 1982), and then treated according to the respective metal analysis.

In tissues, iron was determined with 1,10-phenanthroline (Sandell, 1959; Fries, 1972), and Ti and V were separated (Korkisch, 1969; Fukasawa and Yamane, 1977) prior to their determinations. Titanium was determined according to Qureshi *et al.* (1968), and vanadium using the methods of Bannard and Burton (1968) and Fukasawa and Yamane (1977). In the fractions, iron was determined using 2,4,6-tri-2 pyridyl-1,3,5-triazine (Collins *et al.*, 1959; Box, 1981), and vanadium and titanium as above, without separating them after digestion of the fractions with a binary $HNO_3/HClO_4$ acid system (Jones *et al.*, 1982).

Blank controls were used in every metal analysis, and except in the fractions, all the determinations were performed in triplicate.

Determination of the major ionic composition and relative reduced feature of the fluids

Chlorinity and salinity were determined conductimetrically with respect to standard seawater at 25°C (conductimeter Radiometer CDM 2e, with a standard cell CDC 104). Chloride was determined by Mohr titration and sulphate by direct titration with barium perchlorate using Thorin as indicator. Subsequently, cations were removed by passing the sample through a strong acid cation exchange resin column (Fritz and Yamamura, 1955), except in seawater in which case sulphate was determined gravimetrically as $BaSO_4$. Successive determination of calcium and magnesium were made by potentiometric titration with a calcium ion selective electrode (Roman *et al.*, 1982); Na, K, and Li analysis were performed by flame emission spectrophotometry on a Radiometer FLM-3; pH measurements were made potentiomatrically on a Radiometer pH Meter 26 with glass membrane electrode. All major component determinations were made in triplicate.

The relative reduced feature of the plasma and cytosolic solutions were tested by two redox potentiometric titrations (non-standard biochemical methods). In the first, aliquot samples (10–20 ml) in polypropylene vessel were put into a Radiometer TTA-80 titration assembly, acidified with 0.75% HCl, and then titrated with a standard solution 0.1 *N* $KMnO_4$. In the second, aliquot samples (10–20 ml) were acidified with 2 ml of concentrated $HClO_4$, treated with 5 ml of a standard solution 0.1 *N* $K_2Cr_2O_7$, and then titrated with a standard solution of Fe(II).

Chromatographic fractionation of blood plasma

Fractionations were performed on Sephadex G-75 and LH-20 gels, in thermostated chromatography columns (Pharmacia Fine Chemicals K26/40) loaded with 4 g of Sephadex G-75 and 13 g of Sephadex LH-20, respectively. The column temperature was 20°C, but all samples and eluants were cooled at 4–5°C. Plasma samples were concentrated by freeze-dry (Freezer-dryer-5 Labconco), five-fold for *P. chilensis* and two-fold for *A. dispar* before running the chromatography procedures. The void volume of the column (V_0) was determined using blue dextran-2000, and the bed volume (V_t) was calculated according to the height and diameter of the gel column.

In G-75 chromatography the sample volumes were 6 and 10 ml for *A. dispar* and *P. chilensis;* the eluants were 0.01 *M* NaCl and 0.06 *M* acetic acid, respectively, cooled and deaereated, collecting fractions of 10 ml (plasma of *A. dispar*) and 6 ml (plasma of *P. chilensis*). The absorbance at 278, 288, 310, 375, 454, and 675 nm (plasma of *P. chilensis*), 265 and 322 nm (plasma of *A. dispar*), and the metal analysis in all fractions were monitored with respect to eluant solutions, previously passed through the respective column, as reference or blank, respectively.

In LH-20 chromatography, to minimize inhomogeneities in the column the gel was packed after swelling in deaereated methanol. One bed volume column of each of the following eluants: water, 25, 50 and 75% methanol in water (v/v), were then passed through the column, followed by 99.8% methanol, collecting two 10 ml fractions per eluant for use as reference or blank solutions. The column was then loaded with the sample (10 ml). Chromatography was performed using 1.5 bed volume of each cooled deaereated methanol/water gradient from 0 to 99.8% methanol according to Macara *et al.* (1979b), collecting 6 ml (plasma chromatography of *P. chilensis*) and 10 ml (plasma chromatography of *A. dispar*) fractions. Absorbance at 272, 288, 310, 320, 375, 454, and 675 nm (plasma of *P. chilensis*), 266, 280, 326 and 660 nm (plasma of *A. dispar*), and metal analysis in all fractions were monitored. Ultra-violet and visible spectra were recorded for whole plasma and the peak-fractions from the eluting patterns, employing a Beckman 35 spectrophotometer. All other absorciometric measurements were also made using this instrument.

Results

Metal analysis

Metal concentrations found on specimens and various tissues of tunicates are listed as mg/Kg dry weight (Table I). Concentrations for plasma are given in mg/l. Ni, Co, Mn, and Nb were not detected in blood cells. Higher concentrations of iron and titanium, and iron, titanium, and vanadium were found in *P. chilensis* and *A. dispar* blood cells, respectively. Although V was not detected in *P. chilensis* blood cells and was found in *A. dispar* blood cells, only trace levels of it were found in both blood plasmas. Ti was not detected in *P. chilensis* blood plasma.

Results of the metal analysis in cell lysate (cytosolic solution), calculated by the difference between the metal contents in whole blood cells and in blood cell residues, are tabulated as percentage of metals in Tables IIa, b, c, respectively. Here, the cellular residues were not washed with acid prior to analysis. Aqueous and 0.75% HCl/methanol cell lysis procedures were considered (*P. chilensis*). These results show that iron content in cell residues from aqueous and HCl/methanol cell lysis procedures are low and comparable, but the titanium content, surprisingly, was higher and greater in the cellular residues than in the cytosolic solution for both cell lysis procedures, but higher in cell residues from HCl/methanol lysis method. Therefore, it is possible that metallic precipitation by extensive hydrolysis (Agudelo *et al.*, 1983b,

Table I

Relative distribution of Fe, Ti, and V contents (mg/kg dry) in Pyura chilensis *and* Ascidia dispar

	P. chilensis			*A. dispar*		
	Fe	Ti	V	Fe	Ti	V
Specimen	191.8	n.d.	n.d.	94.1	107.2	25.7
Body (without tunic)	84.3	n.d.	n.d.	717.4	53.1	25.4
Siphons	70.7	n.d.	n.d.	86.4	16.2	34.8
Tunic	243.9	n.d.	1.5	74.3	125.6	0.4
Blood plasma	45.0	n.d.	1.9	93.7	61.4	22.0
	[a](1.5	n.d.	0.06	2.9	2.1	0.8)
Blood cells	1,105.4	277.8	n.d.	2,181.5	1,552.5	692.9
Blood cell residues[b]	7.4	132.8	n.d.	586.4	784.1	163.7
Blood cell residues[c]	17.3	258.9	n.d.	d	d	d

[a] mg/l; [b]from lysed cell preparations produced by subjecting the cell samples to several freeze-thaw cycles in deionized water media and then centrifuging them at 8000 rpm; [c]from lysed cell preparations produced by subjecting the cell samples to treatment with methanolic solution of 0.75% HCl centrifuging them at 8000 rpm; [d]not determined; n.d. = not detected.

1985) may have been minimized during water cell lysis in the conditions of this work. Thus, it appears that more attention should be focused on tunicate blood cell lysis procedures.

Table IIa

Relative iron distribution in blood cells as determined in pooled samples

Species	Cytosolic[d,e] solution	Cell residues
P. chilensis	99.3%	0.7%
P. chilensis	98.4%[f]	1.6%[f]
A. dispar	73.1%	26.9%

Table IIb

Relative titanium distribution in blood cells as determined in pooled samples

P. chilensis	52.2%	47.8%
P. chilensis	6.8%[f]	93.2%[f]
A. dispar	49.5%	50.5%

Table IIc

Relative vanadium distribution in blood cells as determined in pooled samples

A. dispar	76.4%	23.6%

[d] Calculated by difference between contents in whole blood cells and in blood cells residues (Table I); [e]in respect to lysed cells preparations produced by subjecting the cell samples to several freeze-thaw cycles in deionized water media/centrifuging them at 8000 rpm; [f]with respect to lysed cell preparations produced by subjecting the cell samples to treatment with a methanolic solution of 0.75% HCl/centrifuging them at 8000 rpm.

Ion composition and reduced tendency of blood fluids

The pH and ionic composition of plasma, lysed whole blood, and cytosolic solutions, for both species, are shown in Tables III and IV. The sulphate content in *A. dispar* plasma was greater than in *P. chilensis*, but both contents were lower than in seawater. In the cytosolic solutions, *e.g.*, aqueous intracellular media, the concentrations of sulphate were low with respect to the plasma. Calcium and magnesium contents in *P. chilensis* plasma are higher than in *A. dispar*. In *P. chilensis* some enrichment occurred with respect to seawater, which also occurs for sodium and potassium. Calcium, magnesium, sodium, and potassium contents also were lower in whole lysed blood than in plasma (*P. chilensis*). In *P. chilensis* blood cells, the sodium concentration in the cytosolic solution is only 50% of the *A. dispar* cytosolic solution. However, potassium concentration is very low.

The pH of the whole lysed blood (*P. chilensis*) was nearly alkaline, the salinity almost equal to the seawater from which the specimens were obtained. The sulphate concentration was only 62% of its concentration in blood plasma.

Plasmas, 0.75% HCl/methanolic extracts from blood cells, and cytosolic solutions had reducing tendency in both species in respect to dichromate and permanganate, respectively.

Spectral-separative chromatographic behavior of iron, titanium, and vanadium in plasma

P. chilensis plasma is pink-orange and *A. dispar* plasma is greenish-yellow. Figure 1 shows the UV-visible spectra of both species' plasma. The bands 265–290, 300–330, and 675 nm regions were common to both

Table III

Ionic composition of the blood plasma of Pyura chilensis *and* Ascidia dispar

	Plasma *P. chilensis*	Plasma *A. dispar*	Surface coastal seawater[a]
Chlorinity ‰	19.38	18.64	19.44
Salinity ‰	32.35	33.67	35.13
Cl^- (g/l)	19.86	19.05	19.51
SO_4 (g/l)	0.60	0.79	2.60
Ca^{2+} (g/l)	0.51	0.34	0.44
Mg^{2+} (g/l)	1.54	1.14	1.33
Na^+ (g/l)	15.68	9.34	11.16
K^+ (g/l)	0.78	0.42	0.40
Li^+ (mg/l)	0.87	0.84	1.32
pH	6.77	6.48	8.03
Na/K	0.33	0.30	0.23
Ca/Mg	20.10	22.20	27.90

[a] Surface coastal seawater of Bahía Mejillones del Sur.

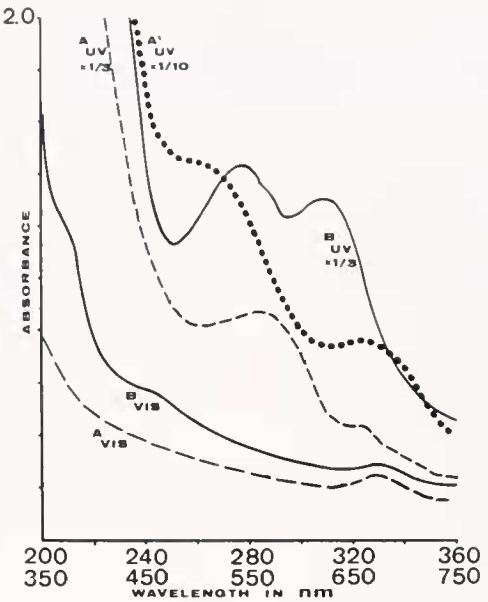

Figure 1. Ultraviolet-visible spectra of blood plasma of *Ascidia dispar* (concentrated twofold by freeze-dry and acidified at pH 3 with acetic acid, that also was the reference solution A_{UV} ···; fresh, water as reference $A_{UV,VIS}$ – – –), and *Pyura chilensis* (fresh, water as reference $B_{UV,VIS}$ solid line). Cell pathlength 1 cm. Dilution shown were applicable.

spectra, with a light bathochromic effect in the UV bands of *A. dispar* plasma with respect to the *P. chilensis* UV spectrum of plasma, which also shows a shoulder in the 280–290 nm zone.

The elution patterns detected at 265 nm for *A. dispar* and at 310 nm for *P. chilensis* are given in Figure 2a. None of the *P. chilensis* fractions were colored, but fractions 9–11 were yellowish in *A. dispar* plasma chromatography. The elution profiles of iron and vanadium for *P. chilensis*, and iron, titanium, and vanadium for *A. dispar* are also presented in Figure 2b. In both species patterns, two peaks were obtained with respect to absorbance, each one in fractions 3, 4; 6–9 (*A. dispar*), and 5, 6; 10–12 (*P. chilensis*). The first band eluted was in the void volume of the column (V_0 = 30 ml) and should have contained compounds with greater molecular weight or at least comparable to the upper exclusion limit of the G-75 column bed. The second band eluted was at a greater volume than V_t(V_e = 61 and 67 ml for *P. chilensis* and *A. dispar* plasmas, respectively) and should have contained compounds with less molecular weight or comparable to the lowest exclusion limit of the G-75 column bed. This also should be valid for the yellow fractions (9–11) from *A. dispar* plasma chromatography. The absorbance profile at 322 nm showed equal characteristics for *A. dispar*, and the same occurred with the profiles at 278, 288, 375, 454, and 675 nm for *P. chilensis*.

Four peaks were obtained for *P. chilensis* with respect to the iron content in fractions, whose elution volumes (V_e) were 13, 25, 55, and 67 ml. The second peak had the same values of the chromatographic behavior parameter (V_e/V_o, V_e/V_t, K_{av}) of the first band in function of absorbance at 310 nm, and so on. These fractions (5, 6) should have contained iron compounds of high molecular weight, found for the first iron band. The other peaks should correspond to iron compounds of low molecular weight. Vanadium was also eluted after the bed volume.

Table IV

Ionic composition of lysed whole blood and cytosolic solutions of Pyura chilensis *and* Ascidia dispar[a]

	Lysed whole blood of *P. chilensis*[b]	Cytosolic solution of *P. chilensis*[c]	Cytosolic solution of *A. dispar*[c]
Chlorinity ‰	19.74	n.m.	n.m.
Salinity ‰	35.66	n.m.	n.m.
Cl^- (g/l)	19.57	n.m.	n.m.
SO_4 (g/l)	0.37	0.070	0.051
Ca^{2+} (g/l)	0.30	n.d.	n.m.
Mg^{2+} (g/l)	1.03	n.d.	n.m.
Na^+ (g/l)	9.59	0.051	0.104
K^+ (mg/l)	484.90	11.20	0.30
Li^+ (mg/l)	0.94	n.d.	0.02
pH	7.82	7.01	7.36
Na/K	0.29	4.6	346.7
Ca/Mg	19.8	—	—

[a] Analysis on lysed whole blood of *A. dispar* were not made due to lack of samples; [b]from subjecting the samples of blood to several freeze-thaw cycles and then centrifuging at 8000 rpm; [c]from subjecting the cell samples to several freeze-thaw cycles with deionized water and then centrifuging them at 8000 rpm; n.m. = not measured; n.d. = not detected.

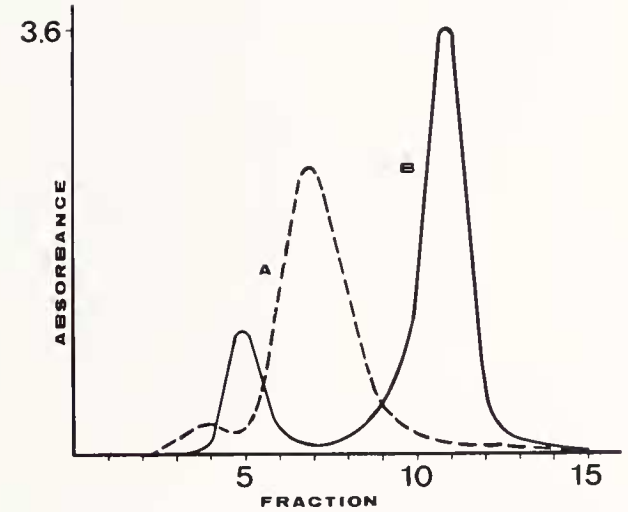

Figure 2a. Elution patterns of blood plasma of *Ascidia dispar* from Sephadex G-75 chromatography at 270 nm (6 ml concentrated twofold by freeze-drying, 6 ml fractions, A dash line), and *Pyura chilensis* at 310 nm (10 ml concentrated fivefold by freeze-drying, 6 ml fractions, B solid line).

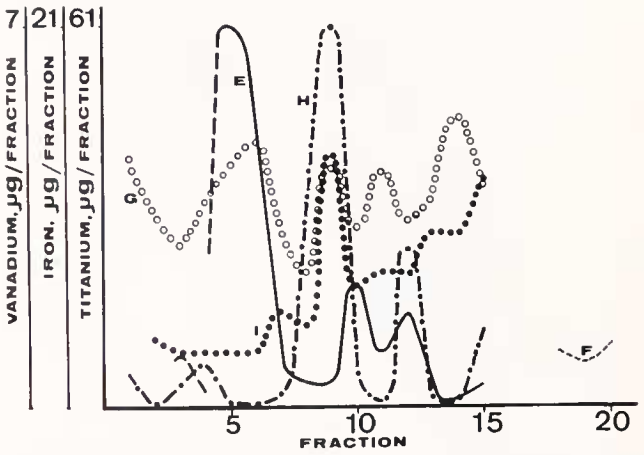

Figure 2b. Elution patterns of metal contents per fraction from Sephadex G-75 chromatography: in plasmas of *Pyura chilensis* (iron E solid line; vanadium F~), and *Ascidia dispar* (iron G open circles; titanium H –·–·–; vanadium I closed circles). Conditions, samples, and fraction volumes are of Figure 2a.

Four bands were also obtained for *A. dispar* with respect to iron content in fractions (V_e = 57, 87, 107, and 137 ml). None had the same values of the chromatographic parameters of the bands in function of absorbance at 265 nm. The four V_e values are greater than the V_t, therefore they should not contain iron compounds of high molecular weight. However, for titanium (three bands, V_e = 37, 87, and 117 ml) the first peak is superposed and similar in the profile at 265 nm, which should mean that it corresponds to titanium compounds with a high molecular weight. The other bands are after the bed volume. The eluted vanadium show increasing contents after fraction 10, for which only two bands were considered (V_e = 67 and 87 ml), both after the bed volume, where the first is superposed with the second peak at 265 nm.

The elution profiles for *P. chilensis* and *A. dispar* blood plasma chromatography on Sephadex LH-20, employing methanol/water gradient as eluants are given in Figures 3 and 4. None of the fractions were colored. At 272 nm, two major bands and one shoulder were obtained for *P. chilensis*, each in fractions 6–9, 11–13, and 14–15. At 310 nm, three bands and two shoulders were obtained, each in fractions 6–9, 11–12, 14–15, and 18–19, respectively. Profiles were also detected at 288 nm (which is superposed with the profile at 272 nm), 320, 375 nm (which were superposed with the profile at 310 nm), and at 454, and 675 nm, which were superposed between them (no bands were obtained in fractions 5–6, 7–8, 10–11, and 15–16).

In *A. dispar*, 6 peaks and 1 shoulder were obtained at 266 nm, each in fractions 4–6, 9, 15–16 (shoulder), 21–22, 25, 29, and 34 (small). At 326 nm one major band was obtained (fractions 3–6), although two small peaks were also observed at fractions 29 and 34, respectively. In addition, patterns were detected at: 288 nm (that was not superposed with the profile at 266 nm, only for the shoulder, fraction 11) and at 660 nm (no bands were obtained in fractions 2–3 and 33–34).

Iron was eluted in all LH-20 chromatography of *P. chilensis* plasma. The V_e of the main bands were at 49, 61, 85, 97, 115, 133, and 235 ml. The first three bands were superposed with the respective eluting peaks at 272 nm, and also with three eluting bands of the profile at 310, and with two peaks of eluting profile at 675 nm. Most of the main iron bands in the profiles were observed at a greater volume than V_t of the bed column, and after fraction number 20, appeared not to have association with the patterns at 272, 288, 310, 375, 454, and 675 nm. Vanadium was not considered in this opportunity.

Iron was also found in all LH-20 chromatography of *A. dispar*, and the V_e of the main peaks were obtained at 35, 55 (shoulder), 115, 145, 165, 195, 215, 265, 295, and 330 ml, in which the chromatographic parameters of any of them correlates with the eluting peaks with respect to absorbance eluting patterns. Titanium was not found in fractions 7–14, and the V_e of the main bands were obtained at 45, 155, 185, 205, 265, and 305 ml. The second titanium eluting band correlates with the respective peaks in the profile at 266 nm, and the fourth is superposed with the patterns at 266 and 280 nm. Vanadium was found in all the chromatography, but most was

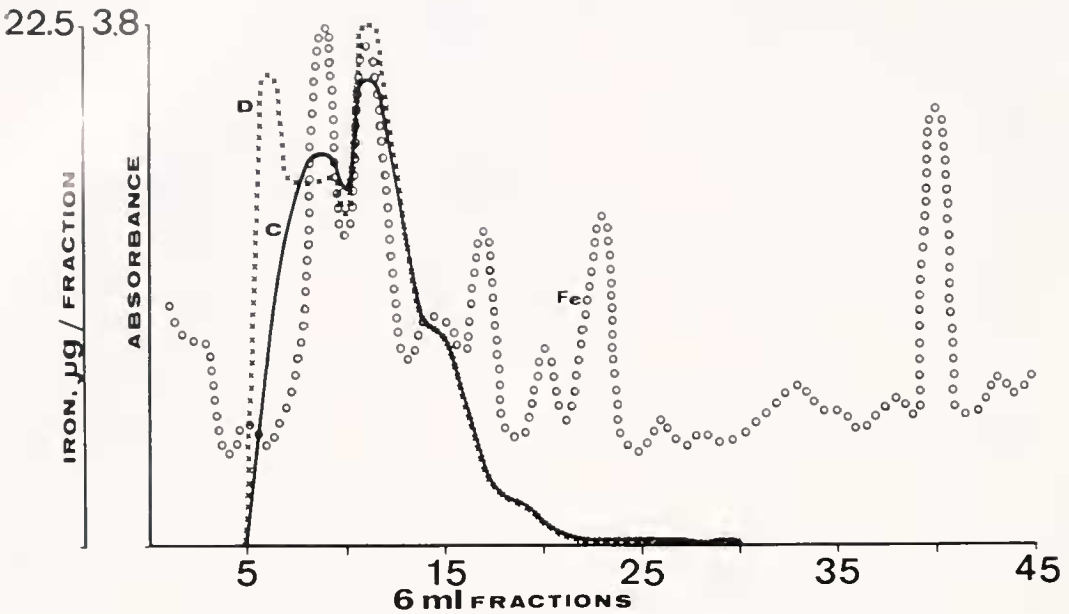

Figure 3. Elution patterns of blood plasma of *Pyura chilensis* from Sephadex LH-20 chromatography at 272 nm (C solid line) and 310 nm (D × × ×), and elution profile of iron (circles). 10 ml concentrated fivefold by freeze-drying, 6 ml fractions.

eluted from fractions 1–17 (V_e = 5, 25, 75, 95, 115, 145, 265, 285, and 335 ml).

Fractions 5 and 11 absorption spectra from Sephadex G-75 chromatography of *P. chilensis* blood plasma are shown in Figure 5a. Fraction 5, that also corresponds to the second iron-band in the respective eluting profile (Fig. 2b), had an absorption band at 276 nm with one shoulder at 400–425 nm. Fraction 11 shows absorption maxima at 270, 310, and 460 nm with a shoulder at 360–375 nm, and is not in the area of an iron-band, although it is between the third and fourth iron-band, in the respective eluting pattern (Fig. 2b).

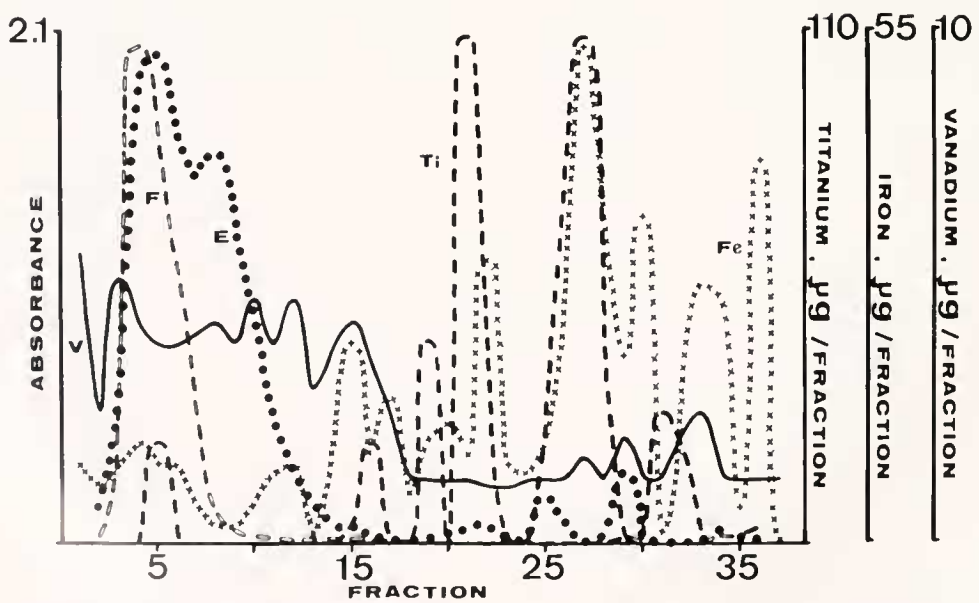

Figure 4. Elution patterns of blood plasma of *Ascidia dispar* from Sephadex LH-20 chromatography at 266 nm (E dots) and 326 nm (F ○ ○ ○), and elution profiles of iron (× × ×), titanium (dash line), and vanadium (solid line). 10 ml concentrated twofold by freeze-drying, 10 ml fractions.

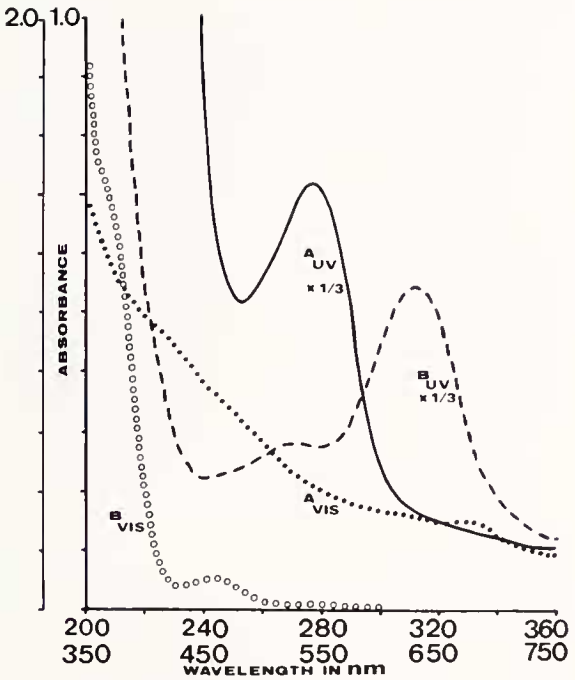

Figure 5a. Absorption spectra of fractions 5 (A_{UV} solid line, A_{VIS} dots) and 11 (B_{UV} dash line, B_{VIS} circles) from Sephadex G-75 chromatography of blood plasma of *Pyura chilensis*.

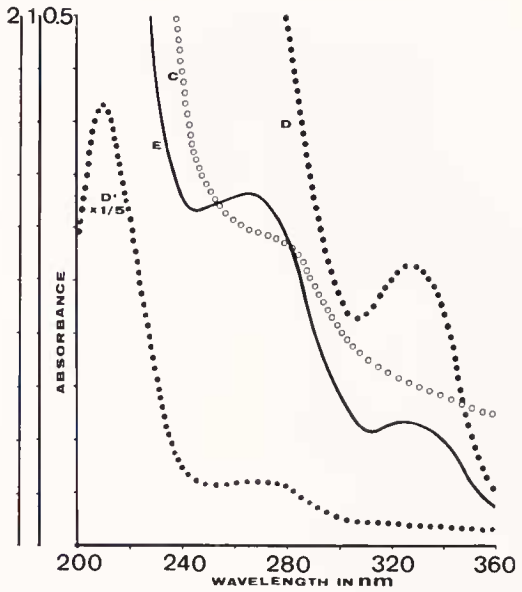

Figure 5b. Ultraviolet spectra of fractions 4 (C circles), 7 (D, D' dots), and 11 (E solid line) from Sephadex G-75 chromatography of blood plasma of *Ascidia dispar*. Dilution shown were applicable.

Ultraviolet spectra of fractions 4, 7 and 11 from Sephadex G-75 chromatography of *A. dispar* blood plasma are shown in Figure 5b. Fraction 4 had an absorption shoulder at 265–285 nm and also corresponds to the first titanium-band in the respective profile (Fig. 2b). Fraction 7 shows absorption bands at 210, 260–280, and 326 nm, and it corresponds to the first vanadium band (Fig. 2b). Fraction 11 (yellowish) had two absorption maxima, at 266 and 326 nm, respectively, and corresponds to the third iron band (Fig. 2b).

Ultraviolet spectra of fractions 5, 8, 17, 21, 25, 29, and 36 from Sephadex LH-20 chromatography of *A. dispar* blood plasma are shown in Figure 6a. Fractions 3–5 had absorption maxima at 266–270 and 322–324 nm, corresponding moreover to the border-line zone between the first iron band and the respective iron shoulder, and to the first titanium band (Fig. 4). Fraction 8 also had two absorption bands, at 262 and 320 nm, which only appear to be associated with the third vanadium peak (Fig. 4). Fraction 17 had ultraviolet bands at 232 nm and in the zone of 280 nm, corresponding to the fourth iron peak in Figure 4. Fraction 21 had one absorption band at 280 nm and two small shoulders at 274–276 nm and 286–288 nm, respectively. This fraction also corresponds to the first titanium band (Fig. 4). Fraction 25 had ultraviolet bands at 230 and 270 nm, and one shoulder at 292–294 nm. This fraction appears not to be associated with any metal. Fraction 29 had the following absorption maxima: at 210, 232 (shoulder), 270, and 292–294 nm (shoulder), and should correspond to the same group of compounds as fraction 25 (have similar UV spectra). Fraction 36 had three ultraviolet maxima, at 218 (not shown), 296, and 328 nm, and it corresponds to the last iron band (Fig. 4). Fraction 34 had a spectrum similar to fraction 36, except for the band at 296 nm, which in fraction 34 appears as a shoulder in the zone of 280 nm. Also, fraction 34 correspond to the penultimate iron peak (Fig. 4). The visible spectra of fractions only showed absorption increasing monotonically with a decreasing wavelength.

Ultraviolet spectra of fractions 6, 8, and 11 from Sephadex LH-20 chromatography of *P. chilensis* blood plasma are shown in Figure 6b. Fraction 6 had absorption maxima at 280 nm and in the 310–320 nm zone. It appeared not to be associated with any principal iron band (Fig. 3) although it is in the borderline of a minor iron peak (fraction 5). Fraction 8–9 also had two ultraviolet bands, at 270 and 302–306 nm, but are in the first principal iron peak zone (Fig. 3). Fraction 11 had an absorption shoulder band at 260–280 nm and another that tends to disappear at 286–288 nm. This fraction is in the second principal iron peak zone (Fig. 3). In the 12–24 fraction range, the absorption spectra showed no bands. From fractions 25 to 29, the ultraviolet spectra only showed one light band at 266 nm. The visible spectra of fractions also consisted in absorptions increasing monotonically with decreasing wavelength.

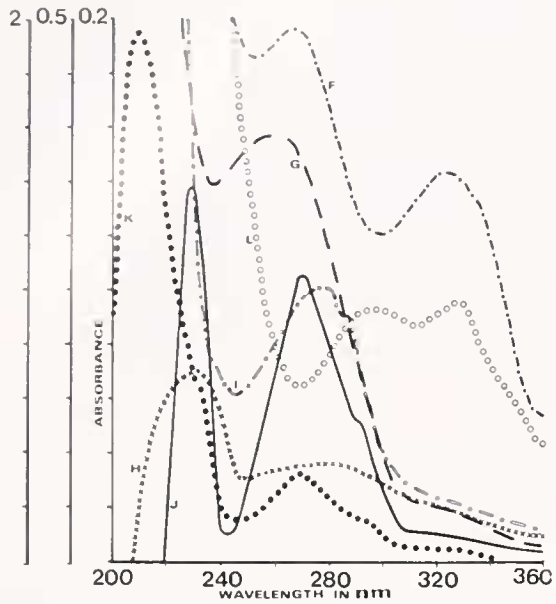

Figure 6a. Ultraviolet spectra of fractions 5 (F –·–·–), 8 (G dash line), 17 (H × × ×), 21 (I ○ □ ○), 25 (J solid line), 29 (K dots), and 36 (L circles) from Sephadex LH-20 chromatography of blood plasma of *Ascidia dispar*.

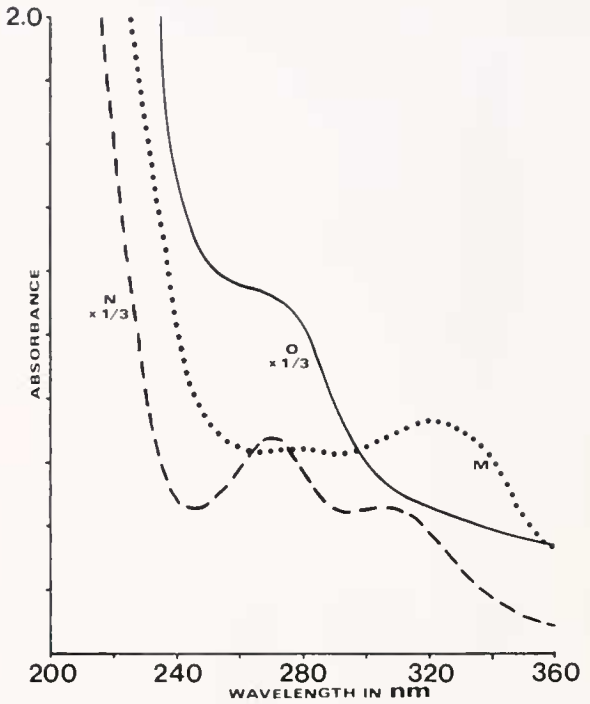

Figure 6b. Ultraviolet spectra of fractions 6 (M dots), 8 (N dash line), and 11 (O solid line) from Sephadex LH-20 chromatography of blood plasma of *Pyura chilensis*.

Discussion

The analysis reported here should support the conclusion that *P. chilensis* is an iron and titanium accumulator, and that *A. dispar* is an iron, titanium, and vanadium accumulator. In both species the predominant metal was iron, which in the case of *P. chilensis* is consistent with ascidian phylogeny with respect to vanadium- and iron-containing species (Hawkins *et al.*, 1983c). In the order Pleurogona, all of its family species are iron accumulators (Swinehart *et al.*, 1974; Agudelo *et al.*, 1982). However, *A. dispar* appears to be an iron-predominant species, although, it also accumulates titanium and vanadium at greater levels than considered non-biological (Saxby, 1969; Hawkins *et al.*, 1983c) with respect to metal contents in blood cells. Results from the whole body (specimens) are not reliable because when the animal is removed it immediately begins to lose blood. In the sub-orders Aplousobranchia and Phlebobranchia, the majority contain vanadium in their blood (Hawkins *et al.*, 1983c; Michibata *et al.*, 1986). Titanium has been reported in *Ciona intestinalis* (Noddack and Noddack, 1939) and *Eudistoma ritteri* (Levine, 1961, 1962a,b), but according to Goodbody (1974), there is no concrete evidence that titanium would be concentrated in blood cells. In the present work evidence is presented of this metal in the blood cells of *P. chilensis* and *A. dispar*.

However, some of these results could be only apparent from the biochemical point of view, because they may be influenced by the ascidians immediate environment *e.g.*, the floating metallic barrels of marine pools where fixation occurs. TiO_2 and Fe_2O_3 are frequently used as pigments in many paints (Orna, 1980). Because of their ability to accumulate metallic trace elements from seawater, tunicates also have been suggested to serve as marine pollution indicators (Papadopoulou and Kanias, 1977). Therefore, the Ti in *P. chilensis*, and the higher concentrations of Fe and Ti in *A. dispar*, may also be associated with this aspect, rather than being considered essential elements subjected to selective accumulation mechanisms. The accumulation of uncommon metals by ascidians in significant concentrations is still an open question. For instance, something similar to what happens to Ti, occurs to Nb (Rayner-Canham, 1984).

Iron is the predominant metal in *P. chilensis* cytoplasm, but in *A. dispar* 26.9% could be in cell membranes. Titanium is almost distributed likewise in both species' cytoplasm and cell membranes. Vanadium is predominant in *A. dispar* cytoplasm cells, although 23.6% could be bound to membrane cells. Therefore, variable fraction of metals, which may depend on the species, are associated with blood cell membranes of tunicates.

Blood plasma of both species were nearly neutral, with a lower salinity than the habitat seawater and with low concentrations of sulphate ions. Besides, the Ca/Mg concentration ratios were greater (0.33 for *P. chilensis* and

0.30 for *A. dispar*) compared with the seawater (0.23). The Na/K concentration ratios were lower (20.1 for *P. chilensis* and 22.2 for *A. dispar*) than in seawater (27.9).

Calcium and magnesium were not detected in the cytosolic solutions, and the Na/K concentration ratios were very different (4.6 for *P. chilensis* and 346.7 for *A. dispar*). Nevertheless, both were nearly neutral and their sulphate ion contents were low, reaching 11.7% and 6.5% of their contents in plasmas of *P. chilensis* and *A. dispar*, respectively. This implies that the low concentration of sulphate in plasmas (in respect to the concentration of sulphate in seawater), is not the result of the accumulation into cytoplasmic blood cell solutions. Considerable controversy still exists on the intracellular pH and concentration of sulphate in the intact blood cells of tunicates (Dingley *et al.*, 1982; Hawkins *et al.*, 1983a; Frank *et al.*, 1986).

To obtain more knowledge about the behavior of some major tunicate blood components, plasma-cell interaction was abruptly induced in the blood itself (due to lack of *A. nigra* blood, this experiment was carried out only with *P. chilensis* blood). Blood cells apparently were not lysed under whole blood lysis procedures, according to microscope observations and to differential UV-spectra of plasma, cytosolic solution and lysed whole blood samples. The results (Table IV), are consistent with the fact that the blood cells of *P. chilensis* are not acidic and it seems that interactions could occur between plasma and cellular compounds, that could account for the decrease of sulphate, calcium, magnesium, sodium, and potassium concentrations in whole lysed blood solution, in respect to their concentrations in blood plasma. Part of these components could be taken up by some compound(s) of the cellular membranes. It is also possible that sulphate, calcium, and magnesium in particular, interact with some intracellular compounds, which would mean, for instance, that sulphate is consumed by intracellular compounds of cytosolic solutions. Due to the complexometric titration method by means of which calcium and magnesium were determined (Roman *et al.*, 1982), it is feasible that intracellular strong metal ligands take up part of the calcium and magnesium of the plasma. Therefore, this could be the first evidence of sulphate consumption by blood cell components of tunicates, as hypothesized by Hawkins *et al.* (1983b). It should explain its low concentration in ascidian blood plasma as compared to the blood plasma of other marine animals (Burton, 1973).

Both plasmas and cytosolic solutions were reducing with respect to permanganate and dichromate, respectively. However, deproteinization prior to the titration were not made. However, in the case of the back titration of dicromate method, the sample was acidified with concentrated perchloric acid, a deproteinizant (Carr *et al.*, 1983). In the pioneering studies of Endean (1985a) similar assays were tested, and Muzzarelli (1973) used back titration of dicromate for chitin determination. Hawkins *et al.* (1980a) have detected N-acetylaminosugar compounds in the blood plasma of tunicates. Other reducing components that have been reported in ascidian blood include some reduced form of metals, the tunichrome like compounds and the so called apoferreacids (Macara *et al.*, 1979a, b, c; Agudelo *et al.*, 1982, 1983b, 1985; Hawkins *et al.*, 1983b; Bruening *et al.*, 1985; Frank *et al.*, 1986).

Maintaining iron and vanadium in reduced forms in specialized blood cells, and also in some extension in the plasma in the case of iron (Agudelo *et al.*, 1983b; Roman, unpub. results from *P. praeputialis*), required more investigation in adequately controlled artificial conditions.

The plasma spectra (Fig. 1) are similar for *P. chilensis* and *A. dispar*. The main differences are the presence of a shoulder at 375–385 nm, and the existence of pink-orange compound(s) having an absorption band at 450–475 nm in the plasma spectrum of *P. chilensis*. *P. stolonifera* pink compound(s) had a visible band at 497 nm (Hawkins *et al.*, 1980a). The plasmas UV- spectra of *A. nigra* (Kustin *et al.*, 1976), *A. ceratodes* (Hawkins *et al.*, 1980a), *Podoclavella moluccensis, Polycarpa pedunculata* (Hawkins *et al.*, 1980b), and *P. stolonifera* (Hawkins *et al.*, 1980a) also have bands at 260–275 nm and 300–330 nm ranges. A band at 335 nm (Agudelo *et al.*, 1982) was only detected in plasma of *B. ovifera*. The main similarity of the visible spectra of *P. chilensis* and *A. dispar* plasma is the band at the 675 nm zone.

Anion exclusion, cation retardation, and other problems occur in the chromotography of metal-containing substances on Sephadex G and LH types. This is due to the small amounts of donor groups present in the material (Pharmacia Fine Chemicals, 1977; Kura *et al.*, 1977; Johnson and Evans, 1980; Lönnerdall and Hoffman, 1981). To minimize this problem, 0.01 *M* NaCl and 0.06 *M* acetic acid solutions were used as eluents with Sephadex G-75, and methanol/water gradient with Sephadex LH-20 chromatography, respectively. Some level of methanol was always maintained in the separative process and prior to the sample run, the column was conditioned with methanol p.a. As Sephadex LH-20 was used with a mixture of polar solvents, adsorption and partition effects must be considered to play major role in the separation. Gel filtration effects can be disregarded.

The elution behavior of plasmas of *P. chilensis* and *A. dispar* from Sephadex G-75, were similar in respect to absorbance *versus* fraction collected (Fig. 2b), but the patterns for metal contents *versus* fraction collected (Fig. 2b), were not similar in function to the same metal considered. In *P. chilensis* plasma, evidence of iron com-

pounds with a high molecular weight was found (fraction 5–6), in addition to iron bands corresponding to low molecular weight iron compounds. However, these might correspond to iron compounds of high molecular weight that showed a greater affinity for the gel phase than for the aqueous phase. In *A. dispar*, no evidence of high molecular weight iron compounds was found. However, these were found in the case of titanium (fraction 4). Low molecular weight compounds of iron and titanium, or metal compounds that showed greater affinity for the stationary phase were also detected. In both plasmas vanadium appears to exist as low molecular weight compounds, unless the high molecular weight compounds were retarded by adsorption phenomena.

The absorption spectra of the fractions associated with high molecular weight iron compounds (Fig. 5a, fraction 5), cannot correspond to an Fe (III) hydrolytic polymer, which only showed a shoulder at 470 nm (Flynn, 1984). The absorption spectrum of fraction 11 (Fig. 5a) appears to correspond to G-75 low molecular weight organic pigment that could be a tunichrome-like compound(s). The absorption spectra of fractions 5 and 11 account for the spectrum plasma of *P. chilensis*, so these results appear not to be "artifacts."

The ultraviolet spectra of the fractions associated with apparently high molecular weight titanium compounds, from *A. dispar* plasma chromatography on Sephadex G-75 (Fig. 5b, fraction 4), only shows a shoulder at 270–286 nm. This absorption zone was also checked for the indication of a high molecular weight iron compound(s), but no visible bands were observed. The ultraviolet spectra of fractions 7 and 11 (Fig. 5b) appeared to correspond to closely related compounds, apparently of low molecular weight, associated with vanadium and iron, respectively. Their UV spectral features suggest that tunichrome-like compounds may also be involved in these fractions (Bruening *et al.*, 1985). In comparison with the absorption spectrum of the whole blood plasma of *A. dispar* (Fig. 1), in the chromatographic fractions, the absorption peak at 675 nm zone was not observed.

The elution behavior of plasma of *P. chilensis* and *A. dispar* on Sephadex LH-20 with methanol/water gradient, showed similar patterns for absorbance, and iron contents *versus* fraction collected (Fig. 3, 4). For *P. chilensis* plasma, chromatographic evidence of iron-compounds were obtained, and the same occurs for iron, titanium, and vanadium compounds in *A. dispar* plasma, respectively, which appear not to be inorganic hydrolytic products of metal ions.

In *A. dispar*, the absorption spectra of fractions 3–5 (Fig. 6a) appear to be associated with iron and titanium-compounds, but according to the spectra of fractions 4, 7, and 11 from Sephadex G-75 (Fig. 5b), the titanium compound(s) should tend to absorb at 260–290 nm zone. Iron, vanadium-compounds and tunichrome like substances also absorb at 320–330 nm. The ultraviolet spectrum of fraction 8 (Fig. 6a), should correspond then to vanadium compound(s). The ultraviolet spectrum of fraction 17 may correspond to iron compounds of proteinaceous nature, due to the band at 280 nm zone, and the same seems to occur in fraction 21 for titanium compound(s). Fractions 25–29 (Fig. 4) were not associated to any metal ions, and by their spectra appear to correspond to closely related compounds. Fractions 34–36 are related to iron, and by their spectral features should correspond to iron compound(s) similar to those obtained from the interaction between iron and fractions 8–13 G-75 chromatography of *A. ceratodes* plasma (Hawkins *et al.*, 1980a). Therefore, compounds of fractions 3–5 should be closely related to iron compound(s) of fractions 34–36.

In *P. chilensis* plasma chromatography on Sephadex LH-20 gel, fraction 6 (Fig. 3) appear not to be associated with iron, and their spectrum (Fig. 6b) could correspond to tunichrome-like substances similar to spectrum of fraction 11 from Sephadex G-75 (Fig. 5a). However, fractions 8, 9 (Fig. 3) are related to a main iron peak, then those should contain iron compound(s), whose absorption peaks show (Fig. 6b) hipsochromic shifts in respect to the spectrum of fraction 6. Hiper- and hipochromic effects in the bands can also be observed. Fraction 11 is in the zone of the second iron peak (Fig. 3), and by their ultraviolet spectra (Fig. 5a), may correspond to iron compound(s) of proteinaceous nature.

It is likely that by dilution the visible absorption maxima were not observed in the spectra of fractions coming from LH-20 chromatography of blood plasmas.

The complicated hydrolytic processes of iron (Flynn, 1984), titanium (Pascal, 1963, Ciavatta *et al.*, 1985) and vanadium (Kustin and Macara, 1982) in a pH media close to neutrality, such as the blood plasma of tunicates, suggests that these elements could be found as coordination compounds with proteic or non proteic organic ligands. The ligands that have been associated with metals, in tunicate plasma, are proteins (Hawkins *et al.*, 1980a; Webb and Chrystal, 1981; Agudelo *et al.*, 1983b) and N-acetylaminosugar compounds (Hawkins *et al.*, 1980a, b). However, Agudelo *et al.* (1983b) considers that these last compounds could correspond to tunichrome-like substances. It also has been suggested that α-hydroxycarboxylic acids residues could be involved in the metal complexation by tunicates (Rayner-Canham, 1984).

The matter of protein metal-binding, and the study of ligating systems for metals in the blood plasma of ascidians *P. chilensis* and *A. dispar* was scarcely treated here. However, information was obtained about the presence of Fe, Ti complexes, and likely vanadium complexes in blood plasma of species under study. Therefore, it is rea-

sonable to suppose that they are involved in the dynamic processes (storage/carrier) of metals in tunicate blood. Accordingly, the high molecular weight metal compound(s) should be "transferrin"-like metalloproteins, which has been recently shown in the blood plasma of *P. stolonifera* (Martin *et al.*, 1984; Finch and Huebers, 1986).

Between pH 2.5–3.5, tunichrome solutions appear green, due to the broad band in the zone near 660 nm (Macara *et al.*, 1979b). We found an absorption peak around 675 nm in both plasmas and in fraction 5 from Sephadex G-75 chromatography of *P. chilensis* plasma. This should arise from iron-compound(s) of high molecular weight with respect to the exclusion limit of the gel. It has recently been suggested that in the d-d transition energy at 660 nm zone, two nitrogen atoms from the coordination by ligands like D-glucosamine (Micera *et al.*, 1985) could be involved. This was not observed when the amino group was protected, as occurs in the case of N-acetyl-D-glucosamine. Therefore, more attention should be focus in the tunicates blood compounds that show absorption bands at 660–675 nm zone, due to their potential association with metal binding.

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